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METHOD OF PRODUCING HUMAN COAGULATION FACTOR VIII FROM WHOLE PLANTS OR PLANT CELL CULTURES

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FIELD OF THE INVENTION

The present invention relates generally to a method for producing a human coagulation factor VIII from whole plants or plant cell culture. More specifically, the invention relates to a method for producing a human coagulation factor VIII from a whole plant or plant cell culture which results from a plant cell encoded to produce the human factor VIII. This invention is directed to these associated embodiments in all respects. As used herein, the terms "human factor VIII" and "human coagulation factor VIII" are interchangeable and refer to human like proteins possessing human factor VIII sequence identity or human factor VIII-like procoagulant or coagulant activity.

BACKGROUND OF THE INVENTION

Given the technological advances in recombinant DNA technology made over the past decade it has become common practice to introduce new genetic material into plants cells, plants, or plant tissue to establish new traits that enhance the value of the plant or plant tissue. The present invention relates to a method for producing human blood coagulation factor VIII from a

whole plant or plant cell culture which result from a plant cell encoded to produce the human factor VIII.

Factor VIII is a highly specialized type of protein used primarily in the treatment of Hemophilia A as well as for other research and commercial applications. Factor VIII is a large glycoprotein that, when activated, functions in the blood coagulation cascade as a cofactor, along with calcium ions and phospholipid, in the factor IX mediated activation of factor X in the intrinsic coagulation pathway. It can be activated proteolytically by several coagulation enzymes, including thrombin.

Because of the difficulty in its production, plasma-derived factor VIII is in chronically short supply, therefore making this type of therapy highly cost prohibitive. In addition, resulting pharmaceutical products derived from blood plasma are highly impure, with a specific activity of 0.5 to 2 factor VIII units per milligram protein (one unit of factor VIII activity is by definition the activity present in one milliliter of normal plasma). Plasma-derived factor VIII purity is typically lower than 1% by weight (Wood et al. 1984. Nature 312:330). The high level of impurities result in a variety of serious complications including transmission of hepatitis A, B, and C, human parvovirus and human immunodeficiency virus (HIV) pathogens. To circumvent difficulties with viral pathogen transmission associated with human plasma-derived factor VIII and to lower product cost, factor VIII has been successfully expressed in a variety of mammalian cell culture systems. Initially, recombinant factor VIII was produced in baby hamster kidney (BHK) cell lines, using the calcium-phosphate coprecipitation method (Simonsen et al. 1983. Proc Natl Acad Sci USA 80:2495, Wigler et al. 1979. Proc Natl Acad Sci USA 76:1373) for integration of the 7kb protein encoding region (Wood et al. supra 1984, Capon et al. 1997. U.S. Patent No. 5618788). Active recombinant human factor VIII has also been produced in Chinese hamster ovary (CHO) cells (Kaufman et al. 1988. J Biol Chem 263:6352) and monkey COS-7

cells (Toole et al. 1984. Nature 312:342, Truett et al. 1985. DNA 4:333). However, production of factor VIII using these mammalian cell lines does not eliminate the potential for transmission of human pathogens. Consequently, these cell lines require additional quality assurance testing during biosafety trials to safeguard against virus transmission.

Other than the instant invention, production of factor VIII in recombinant hosts other than mammalian cells has not yet been successfully completed. Although factor VIII does not require γ-carboxylation of glutamic acid residues for activity, factor VIII does possess 25 separate glycosylation sites, which may preclude the use of prokaryotic host organisms for its production, especially if glycosylation is necessary for activity. In addition, the large (7.3 kb) protein coding region may preclude the use of many prokaryotic and lower eukaryotic hosts.

General Overview of Transgenic Plants for Foreign Protein Production

Transgenic plants can be used for the production of high value, medicinally important proteins, for example, monoclonal antibodies (Hiatt et al. 1989. Nature 342:76, During et al. 1990. Plant Mol Biol 15:281, Benvenuto et al. 1991. Plant Mol Biol 17:865, Firek et al. 1993. Plant Mol Biol 23:861, Magnuson et al. 1996. Prot Expr Purif 7:220), human growth hormone (Kay et al. 1987. Science 236:1299) and human serum albumin (Sijmons et al. 1990. Bio/Technol 8:217). Transformed cells synthesize, secrete, and accumulate functional antibodies including single (Benvenuto et al. supra 1991) and double (During et al. supra 1990, Hiatt et al. supra 1989) domain immunoglobulins. However, none of these authors reported the production of complex human blood coagulation factors from transgenic plants.

Unlike bacteria, plants perform many of the complex protein-processing steps required to produce mammalian proteins in an active form. Although most mammalian transgene products accumulate in plants at levels below 1% of soluble protein, Hiatt et al. (supra 1989) reported IgG antibody levels of up to 1.3% of soluble protein in tobacco. In addition, foreign protein levels of 14.4% soluble protein were reported in efforts to produce phytase, a digestive enzyme used in

livestock, in transgenic tobacco (Pen et al. 1993. Bio/Technol 11:811). Despite these efforts, there have been no reports of the successful production of any human blood coagulation factors from transgenic plants.

Whole plant and plant cell culture media are well-defined and relatively inexpensive to produce and work with when compared to mammalian cell culture media. Further, plant-based products, unlike mammalian-derived protein formulations, are generally assumed to be neither pathogenic nor oncogenic to humans (Cramer et al. 1996. Ann NY Acad Sci 792:62). Also, when compared to similar production in transgenic bacterial strains (Attaai et al. 1987. Biotechnol Bioeng 30:389), plant transformation methods showed greater stability of foreign gene expression, even without use of selection pressure (Gao et al. 1991. Plant Cell Reports 10:533).

Plants may be transformed to express foreign DNA using a variety of methods including *Agrobacterium* transformation (An 1986. Plant Physiol 81:86-91; Hoekema et al. 1985. Plant Mol Biol 5:8589), microprojectile bombardment (Klein TM, et al., Gene Transfer by Particle Bombardment, Plant Tissue Culture Manual, D1, p. 112, 1991, Kluwer Academic Publishers), pollen transformation (Saunders et al. 1997. U.S. Patent 5629183), chemical mediated uptake by protoplasts (Krens et al. 1982. Nature 296:72-74), and electroporation (Langridge et al. 1985. Plant Cell Reports 4:355-359).

Agrobacterium transformation

A. tumefaciens is the etiological agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms (DeCleene et al. 1976. Bot Rev 42:389). Virulent strains of *A. tumefaciens* contain large, tumor-inducing (Ti) plasmids (at about 200 kb). When wounded plants or plant tissues are cocultivated with such *Agrobacterium* strains, a portion of the Ti plasmid, called T-DNA, is transferred to and integrated into the nuclear genome of the infected plant cells (Hernalsteens et al. 1980. Nature 287:654, Lichtenstein et al. 1987. Genet Eng 6:104). The T-DNA encoded genes are transcribed and translated in the plant tissues, resulting in auxin,

cytokinin and opine synthesis. The elevated auxin and cytokinin levels cause rapid plant cell proliferation, resulting in gall formation (Gheysen et al. 1985. DNA flux across genetic barriers: the crown gall phenomenon, In: Genetic Flux in Plants, Hohn B, et al., Eds., Springer, Wein, pp. 11-49).

To exploit Ti plasmids for genetic transformation, the native T-DNA sequence, responsible for gall formation, can be replaced with foreign DNA, including selectable markers as well as the gene of interest. The only components required for DNA transfer in the T-DNA region are the left and right border sequences (Zambryski et al. 1982. J Mol Appl Genet 1:361). However, since Ti plasmids are difficult to manipulate directly in vitro due to their large size, simplified systems have been developed. The most advanced method utilizes a binary vector system in which the binary vector contains the minimum elements required *in cis* (An supra 1986, An. 1987. Meth Enzy 153:292). Other functions necessary for the gene transfer mechanism are donated from a separate helper Ti plasmid. The binary vector may be directly manipulated in an *E. coli* host and transferred to *A. tumefaciens* containing the helper Ti plasmid, through biparental or triparental mating. Finally, the T-DNA region is integrated into the plant nuclear genome by cocultivation of plant material with transformed *A. tumefaciens*. Representative tissues that have been transformed using an Agrobacterium method include tobacco (Barton et al. 1983. Cell 32:1033), tomato (Fillatti et al. 1987. Bio/Technol 5:726), sunflower (Everett et al. 1987. Bio/Technol 5:1201), cotton (Umbeck et al. 1987. Bio/Technol 5:263), rapeseed (Pua et al. 1987. Bio/Technol 5:815), potato (Facciotti et al. 1985. Bio/Technol 3:241), poplar (Pythoud et al. 1987. Bio/Technol 5:1323) and soybean (Hinchee et al. 1988. Bio/Technol 6:915).

Microprojectile bombardment

In this method, gold or tungsten DNA-coated particles are accelerated towards target plant cells (Klein et al. 1987. Nature 327:70). This technique has been used to obtain stably transformed cultures of maize and tobacco (Klein et al. 1988. Bio/Technol 6:559) as well as

transient expression in onion. A comprehensive summary of microprojectile bombardment is given in Klein TM, et al., Gene Transfer by Particle Bombardment, Plant Tissue Culture Manual, D1, p. 112, 1991, Kluwer Academic Publishers.

Pollen transformation

In pollen transformation techniques, plant germplasm is transformed with foreign DNA by introducing the DNA into pollen grains by a technique such as electroporation (Mishra et al. 1987. Plant Sci 52:135), mating ova of the desired plant line with the transformed pollen, and selecting for the transformed germplasm. The germinating pollen, resulting seed, and the progeny can each be screened for expression of the foreign gene. The transformed pollen can be used as a vector for introducing the foreign DNA into plant lines of similar or dissimilar origin, including both monocots and dicots. To date, pollen collected from tobacco and corn plants has been stably transformed via electroporation (Saunders et al. supra 1997). In addition, tobacco plants have been stably transformed to produce β-glucoronidase by mating ova with transformed tobacco pollen.

Chemical mediated uptake by protoplasts

DNA uptake via chemical stimulation was developed for the direct transformation of both monocot and dicot protoplasts (Krens et al. supra 1982). In this method, the plant cell wall is first degraded enzymatically to form protoplasts, using standard techniques. The protoplasts and vector are then incubated in the presence of polyethylene glycol, which facilitates transformation via direct insertion. Either a direct gene transfer vector or a Ti plasmid may be used in transformation. Since protoplast transformation can facilitate measurable gene expression within 24 to 48 hours, this method has been used widely by many researchers (Pröls et al. 1988. Plant Cell Reports 7:221, Töpfer et al. 1988. Plant Cell Reports 7:225).

Electroporation

Introduction of DNA into plant protoplasts by treatment of the protoplasts with an electric pulse in the presence of the appropriate DNA is a process called electroporation (Fromm et al. 1985. Proc Natl Acad Sci USA 82:5824). Supercoiled or circular plasmid DNA is added to a suspension of protoplasts. The solution is mixed and subjected to a pulse of approximately 400 V/cm at room temperature for less than 10 to 100 μsec. A reversible physical breakdown of the membrane occurs to permit DNA uptake into the protoplasts. The success of the electroporation method is dependent, in part, on optimizing parameters relative to the membrane, the DNA and the electric field. Evidence for the success of transformation after electroporation has been measured by incorporation of radioactively labeled DNA (Tsong et al. 1985. Biblio Haematol 51:108), transient gene expression (Potter et al. Proc Natl Acad Sci USA 1984. 81:7161, Smithies et al. 1985. Nature 317:230), and the formation of stable transformants (Riggs et al. 1986. Proc Natl Acad Sci USA 83:5602, Stopper et al. 1985. Z Naturforsch 40:929). Due to rapid performance associated with electroporation, this technique is often applied for quick isolation and characterization of plant promoters and cis-acting elements (An et al. 1993, Techniques for isolating and characterizing plant transcription promoters, enhancers, and promoters. In: Methods in Plant Molecular Biology and Biotechnology, Glick BR, et al., Eds., CRC Press, Boca Raton, pp. 155-165).

Plant (material) regeneration methods

After transformation of plant tissues, plants must be regenerated for characterization of stable transformants, selection, breeding and segregation analysis, and foreign protein (blood coagulation factor) production. Plants may be regenerated from a variety of tissues including callus culture, protoplasts, and *A. tumefaciens* tissues (i.e., explants or calli). Regeneration from callus tissue has been demonstrated in monocots, such as corn, rice, barley, wheat and rye and dicots, such as sunflower, soybean, cotton, rapeseed and tobacco.

Regeneration of plants from protoplasts is particularly useful for tissues transformed via direct gene transfer methods including electroporation, PEG-mediated transformation, or microparticle bombardment. Regeneration of plants from protoplasts has been demonstrated for rice (Abdulah et al. 1987. Bio/Technol 4:1987), tobacco (Potrykus et al. 1985. Mol Gen Genet 199:169), rapeseed (Kansha et al. 1986. Plant Cell Reports 5:101), and potato (Tavazza et al. 1986. Plant Cell Reports 5:243), among others. Regeneration of plants from tissue transformed with *A. tumefaciens* has been demonstrated for several species of plants including tobacco (Horsch et al. 1985. Science 225:1229, Hererra-Estrella et al. 1983. Nature 303:209), sunflower (Everett et al. supra 1987), tomato (Fillatti et al. supra 1987), rapeseed (Pua et al. supra 1987), and cotton (Umbeck et al. supra 1987), among others.

SUMMARY OF THE INVENTION

The production of factor VIII in transgenic plant systems has the potential to significantly reduce the cost and safety risks associated with current production methods for this product. For example, direct production costs for whole plant processes at equal protein production rates appear to be two to four orders-of-magnitude lower than comparable mammalian cell processes. As plant expression levels increase, this type of production becomes even more capital cost-effective. Additionally, economic analysis indicates that regulatory costs associated with plant cell culture may reduce by as much as $70,000 per batch as compared to analogous mammalian cell processes because plants are regarded as free of human viral pathogens (Crawford. 1995. Therapeutic protein production using plant cell culture. Report by Lasure and Crawford, Inc., Seattle, Washington).

It is, therefore, an object of the present invention to provide whole plant and plant cell culture derived human coagulation factor VIII at lower cost when compared to mammalian host cell systems.

It is another object of the present invention to use *Nicotiana tabacum* (tobacco) and other plant species including those representing monocotyledonous and dicotyledonous plants to produce factor VIII.

According to the present invention, the production of human coagulation factor VIII is achieved in whole plants or plant cell culture wherein the human coagulation factor VIII is produced by the methods disclosed resulting in human factor VIII coagulant antigen (FVIII:ag) appearing to be correctly processed *in planta*.

Modifying encoding sequences and subcloning into a plant expression vector are done using standard molecular cloning procedures (Ausubel et al. 1992. Current protocols in molecular biology. Wiley, New York) and splicing PCR techniques (Marks et al. 1992. J Biol Chem 267:16007).

The success of the present invention is evidenced by:

(a) Immunological cross-reactivity of antibodies raised against plasma-derived factor VIII proteins with clone-derived factor VIII proteins as validated by enzyme-linked immunosorbent (dot blot) assay.

(b) Comparable relative protein size between the clone-derived factor VIII proteins and the plasma-derived factor VIII proteins as validated using Western blot immunoassay.

Thus, the present invention is based upon the successful use of plant molecular biology methods to produce human factor VIII *in planta*.

The subject matter of the present invention is particularly pointed out and distinctly claimed in the concluding portion of this specification. However, both the organization and method of operation, together with further advantages and objects thereof, may best be understood by reference to the following description taken in connection with accompanying drawings wherein like reference characters refer to like elements.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts schematically the construction of pSP64-FVIIIc, a vector used to obtain the encoding sequence for pre-coagulation factor VIII for cloning into the plant expression vector pGA748. Pre-coagulation factor VIII cDNA encoding the 2332 amino acid mature protein plus the 19 amino acid native signal was inserted into the pSP64 plasmid at the Sal I site using a Sal I adapter, resulting in the new 10200 bp pSP64-FVIIIc plasmid. This plasmid carried the ampicillin resistance gene for positive transformant selection in *E. coli*.

FIG. 2 depicts schematically the construction of pZD201, the plant expression vector carrying the pre-coagulation factor VIII encoding region. The full length pre-coagulation factor VIII cDNA was excised with Sal I restriction enzyme and sequentially ligated into the compatible restriction enzyme site Xho I located between the CaMV 35S promoter and T7-T5 transcript terminator of binary vector pGA748, forming the 18800 bp plasmid pZD201. This plasmid carried the tetracycline resistance gene for positive transformant selection in *E. coli* and *Agrobacterium tumefaciens* as well as the kanamycin resistance gene for selection of whole plant and plant cell culture positive transformants.

FIG. 3 shows the result of dot blot immunoassay for T0 Factor VIII plant transformants. Positive control plasma-derived factor VIII standards (American Diagnostica, Greenwich, CT) are shown as S1 and S2. Negative control leaf protein extracted from untransformed *Nicotiana tabacum* cv. SR1 is shown as SR. Positive plant primary transformants (leaf protein extracts) are shown as 1004-3, 1006-2 and 1006-3.

FIG. 4 shows protein gel bands resulting from Western blot immunoassay completed on protein extracts from several plant transformants as compared to a non-transformed control culture and plasma-derived factor VIII standard (American Diagnostica, Greenwich, CT). Lane

1 shows the plasma-derived factor VIII (FVIIIc) standard; lane 2 shows total protein extracts from leaf explants taken from an untransformed *N. tabacum* cv. SR1 control; lanes 3 through 9 show total protein extracts from leaf explants taken from T1 plant lines derived from primary transformant tobacco plants.

DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

The present invention is a method for production of human coagulation factor VIII using whole plants as well as plant cell suspensions transformed with appropriately constructed vector plasmids. More specifically, the method of the present invention is stable expression of human coagulation factor VIII:ag, that exhibits immunological cross-reactivity with antibodies to human factor VIII. It is comtemplated that virtually any plant from the plant kingdom may be utilized. Specific types of plants that are amenable to the transformation steps disclosed herein include, but are not limited to monocotyledonous and dicotyledonous plants. Example species include but are not limited to *Nicotiana tabacum* (tobacco), *Solanum tuberosum* (potato), *Glycine max* (soybean), and *Zea mays* (corn).

The method of the present invention for producing a human coagulation factor VIII from plant cells, comprises the steps of:

(a) introducing an encoding sequence for production of human coagulation factor VIII into a plant expression vector in the plant cells;

(b) obtaining a positive transformant of the plant cells, which carries genetic material encoding the production of the human coagulation factor VIII;

(c) cultivating the positive transformant; and

(d) obtaining the human coagulation factor VIII.

The step (b) may be quite simple (such as purchasing transformants) or relatively more complex, resulting from actual transformation by well known methods, for example direct particle bombardment as described in Gene Transfer by Particle Bombardment, Klein TM, Knowlton S, Arentzen R, Plant Tissue Culture Manual, D1, pp 1-12, 1991, Kluwer Academic Publishers, or by Agrobacterium mediated transformation as described in Hoekema et al. (supra 1985), and further described herein.

The step (c) of cultivating involves either whole plant cultivating or tissue cultivating by any of several well known cultivating methods.

The step (d) of obtaining the factor VIII is by well known separation and purification steps, for example protein precipitation, ultrafiltration, affinity chromatography, and/or electrophoresis.

An agrobacterium mediated transformation method of the present invention comprises the steps of:

(a) modifying the coagulation factor VIII encoding sequence for subcloning into a plant expression vector,

(b) subcloning the encoding sequence into the plant expression vector;

(c) transferring the plant expression vector to agrobacterium;

(d) co-cultivating a portion of the transgenic plant cells (suspension culture or leaf disks) with the agrobacterium;

(e) selecting positive transformants from the co-cultivated culture on an antibiotic selective media;

(f) permitting growth of the transgenic plant cells in whole plants or suspensions; and

(g) extracting a liquid containing the human coagulation factor VIII.

Modifying encoding sequences and subcloning into a plant expression vector are accomplished using standard molecular cloning procedures (Ausubel et al. supra 1992) and splicing PCR techniques (Marks et al. supra 1992). More specifically, modifying encoding sequences, comprises the steps of:

(a) adding a transcription promoter to the upstream or 5' end of the factor VIII encoding sequence; and

(b) adding a transcription terminator to the downstream or 3' end of the said encoding sequence. The transcription promoter and the transcription terminator are regulatory elements.

Further, an additional regulatory element encoding a signal peptide may be added between the transcription promoter and the 5' end of the encoding sequence in order to relegate the product human growth factor to a specific cellular organelle. In addition, other regulatory elements may be added either between the promoter and the additional regulatory element encoding the signal peptide or at the 3' end of the encoding sequence to obtain greater mRNA stability between transcription and translation events.

Transferring the plant expression vector into the agrobacterium is completed using the freeze-thaw method (An supra 1987). For monocotyledonous species, super-binary vectors, such as pTOK233 and pSB131, are used to achieve high transformation frequency (Ishida et al. 1996. Nature Biotechnol 14:745). Remaining cocultivation, selection, growth, and extraction steps (d through g) have been described by Magnuson et al. (supra 1996), and are well known in the art of plant molecular biology.

At least one genetic regulatory element may be included in the sequence encoding the transcription of human coagulation factor VIII. Regulatory elements include transcription promoters or enhancers that increase the frequency of transcription events, leader sequences that increase the stability of mRNA prior to translation, and signal peptides that target proteins to

specific organelles for posttranslational modifications and accumulation. Examples of transcription enhancers include but are not limited to the octapine synthase enhancer, a 16 bp palindrome (ACGTAAGCGCTTACGT) (Ellis et al. 1987. EMBO J 6:3203) and the B-domain of the cauliflower mosaic virus 35S promoter (Kay et al. supra 1987). An example of a leader sequence includes but is not limited to alfalfa mosaic virus RNA4 leader sequence (Jobling et al. 1987. Nature 325:622). Examples of signal peptides include but are not limited to the tobacco PR-S signal peptide (Cornelissen et al. 1986. Nature 321:531) and the phytohemagglutinin signal peptide (Hunt et al. 1991. Plant Physiol 96:18).

Example 1

The *Escherichia coli* plasmid pSP64-FVIII (ATCC No. 39812) containing the gene encoding the full-length polypeptide of factor VIII cDNA, derived from human fetal liver, was obtained from ATCC (**FIG. 1**). Coagulation factor VIII is the 2332 amino acid protein which contains a heavy chain, B-domain, and light chain. The 7.2kb pre-coagulation Factor VIII cDNA encodes the native signal peptide (the first 19 amino acid at the N-terminus) as well as the mature protein.

The full length pre-coagulation factor VIII cDNA was excised with Sal I restriction enzyme and sequentially ligated into the compatible restrict enzyme site Xho I located between the CaMV 35S promoter and T7 transcript terminator of binary vector pGA748, forming the 19 kb plasmid pZD201 (**FIG. 2**). This plasmid was directly transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (An supra 1987). The transferred plasmid was introduced into tobacco whole plants (by leaf disks) and calli (by suspension culture) by co-cultivation with agrobacterium thereby producing transformants. Over 200 specific samples of transformants were taken from the co-cultivation and separately placed on kanamycin selective

media. After obtaining positive transformants via kanamycin resistance screening, mature tobacco plants and calli were assayed for the presence of the human coagulation factor VIII. Preliminary protein immunoblotting (dot blot assays-**FIG. 3**) completed using extractable leaf protein showed the presence of coagulation factor VIII antigen in the leaf tissues of T0 whole plant transformants. As seen in **FIG. 3**, strong factor VIII:ag expression characteristics were seen in plants 1004-3, 1006-2 and 1006-3. Positive control factor VIII standards (American Diagnostica, Greenwich, CT) are shown as S1 and S2 whereas negative control leaf protein derived from untransformed *N. tabacum* cv. SR1 is shown as SR. After completion of the dot-blot immunoassay, T0 plants were self-pollenated, resulting in T1 seedstock. T1 seeds were subsequently germinated on kanamycin selective media and mature plants were grown in a controlled environment. Western immunoblot assays shown on **FIG. 4**, completed on leaf protein extracts of T1 plants regenerated from various T0 plant lines, indicate the presence of immunoreactive bands which appear to be comparable in size to those of plasma-derived factor VIII (American Diagnostica, Greenwich, CT). The predominant band appears at approximately 240 kD. Additional faint bands from plasma derived factor VIII and plant transformants appear to correspond to factor VIII heavy chain at 90-200 kD and light chain at approximately 80 kD. The appearance of comparable immunoreactive bands between plant-derived and plasma-derived human factor VIII suggest that plant-derived factor VIII undergoes correct, human-like post-translational modifications. Sheep anti-human factor VIII:C polyclonal antibody (Haematologic Technologies, Inc., Essex Jct., VT) and sheep anti-human factor VIII polyclonal antibody AB787 (Chemicon International, Inc., Temecula, CA) were mixed and used for both the Western blot and dot blot immunoassays.

CLOSURE

While a preferred embodiment of the present invention has been shown and described, it will be apparent to those skilled in the art that many changes and modifications may be made without departing from the invention in its broader aspects. The appended claims are therefore intended to cover all such changes and modifications as fall within the true spirit and scope of the invention.